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Conversion of a mouse Fab into a whole humanized IgG antibody for detecting botulinum toxin

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Abstract. Antibodies serve as the gold standard in most immunodiagnostic assays. Recent advances in recombinant DNA technology have offered the production of antibody fragments or Fabs as promising alternatives. However, the lack of the Fc region of the antibody can be difficult in many standard diagnostic platforms. Therefore we sought to convert a murine Fab into a whole humanized IgG. The variable regions from an anti-botulinum Fab were cloned into human IgG heavy and light chain vectors and produced in myeloma cells. Purified humanized IgG demonstrated conversion to human IgG with no traces of mouse Fab as determined by Western blot analysis. In addition, the humanized IgG performed better as both a detection and capture reagent in an ELISA format and detected the botulinum toxoid at a lower concentration than the parental murine Fab. This technique offers the ability to convert various species of antibodies or antibody fragments into humanized antibodies with improved characteristics in immunodiagnostic assays, for use as human controls in serological assays, or for possible therapeutic benefit.

Keywords: Chimeric antibody, humanized antibody, immunoglobulin expression vector, botulinum neurotoxin

Abbreviations: BoNT, botulinum neurotoxin; BPT, botulinum pentavalent toxoid; Fab, antibody fragment; HRP, horseradish peroxidase; LCκ, kappa light chain; scFv, single-chain antibody fragments; V_L, variable light chain; V_H, variable heavy chain.

1. Introduction

Agent-specific monoclonal and polyclonal antibodies are the most commonly used reagents for affinity-based, antigen-detection assays. They serve as the standard against which other reagents are measured [1]. Traditional techniques for producing these reagents in animals are well established and are used in laboratories throughout the world. While classically derived antibody-based reagents work well in many assays, they are not universally available, easily producible, or suitable for all applications. Polyclonal antibodies often lack the required specificity and monoclonal antibodies may not have sufficient sensitivity to make efficient assays. Often the needs for sensitive and specific antibodies required by advances in immunodiagnostic assays remain unmet. Therefore, there is a growing need to develop alternative approaches for producing high-quality antibody reagents. Traditional animal-based antibody production methods will continue to be required for the near future. However, recombinant DNA technologies offer the promise of improving the existing antibodies and developing new, improved reagents.

Recombinant antibody fragments (Fab) can be expressed, which behave like monoclonal antibodies but consist of only the antigen-binding domains [2]. Commonly, recombinant Fabs are produced by vaccinating

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14. ABSTRACT

Antibodies serve as the gold standard in most immunodiagnostic assays. Recent advances in recombinant DNA technology have offered the production of antibody fragments or Fabs as promising alternatives. However, the lack of the Fc region of the antibody can be difficult in many standard diagnostic platforms. Therefore we sought to convert a murine Fab into a whole humanized IgG. The variable regions from an anti-botulinum Fab were cloned into human IgG heavy and light chain vectors and produced in myeloma cells. Purified humanized IgG demonstrated conversion to human IgG with no traces of mouse Fab as determined by Western blot analysis. In addition, the humanized IgG performed better as both a detection and capture reagent in an ELISA format and detected the botulinum toxoid at a lower concentration than the parental murine Fab. This technique offers the ability to convert various species of antibodies or antibody fragments into humanized antibodies with improved characteristics in immunodiagnostic assays, for use as human controls in serological assays, or for possible therapeutic benefit.

15. SUBJECT TERMS

Clostridium botulinum, neurotoxin, chimeric antibodies, humanized, immunoglobin expression vector

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a mouse with the antigen of interest and creating a cDNA library from isolated spleen mRNA. The heavy- and light-chain genes from all expressed antibodies can be cloned and combined with phage display techniques to create bacterial clones that each display a unique Fab on the surface [3,4]. The phage display library can be screened against the antigen to enrich for new Fabs that bind specifically and selectively to the target [5,6]. This technology was used to create a library of Fabs directed against botulinum neurotoxin (BoNT) that performed well in diagnostic assays [7]. Multiple Fabs were produced and tested for affinity and specificity. The performance of one Fab, BotFab5, when compared to a traditional mouse monoclonal antibody, performed better in all platforms including surface plasmon resonance (SPR), flow cytometry, antigen-capture ELISA, and hand-held assays [2].

Although Fabs can be easier to produce than whole antibodies, they have shortcomings that restrict their use in immunodiagnostic assays. First, they demonstrate less compatibility with widely employed immunoassay platforms that are based on whole IgG antibodies. Second, antibody fragments tend to be less stable than whole IgG, which is of particular concern when environmental conditions may vary considerably. Third, they lack the ability to serve as positive controls in human serological assays. Therefore, converting a recombinant Fab with favorable diagnostic characteristics to a whole immunoglobulin antibody molecule is desirable.

Cloning the Fab variable heavy- and light-chain regions into an expression vector containing an immunoglobulin constant (C) region could transform a useful Fab into a whole antibody molecule with greater diagnostic utility. There are two systems available: two vector systems that employ separate plasmids for expression of the heavy-chain and light-chain sequences and single vector systems in which both sequences occur on the same plasmid [8–10]. A particularly versatile system is the one developed by McLean and coworkers [9]. This two-vector system contains the cDNA for the heavy or light chain under the control of the cytomegalovirus (CMV) viral promoter, which permits transfection of non-lymphoid cells. The vector's small size and high copy number permit efficient propagation and makes this system ideal for expressing Fabs as whole antibodies.

In this study, we demonstrate the conversion of a recombinant Fab into a whole, humanized IgG molecule that can perform equal to or better than the Fab in diagnostic assays. To demonstrate this concept, we chose to use the BotFab5 reagent for conversion into a humanized IgG antibody by using the two-vector system developed by McLean and co-workers [9]. The new antibody consists of a human IgG backbone with the sequence cloned from the variable region of the BotFab5 being the only murine component remaining. BotFab5 recognizes both BoNT A and B and is versatile and sensitive in various immunodiagnostic assays [2,7]. This study was designed to improve the utility of a highly specific Fab for BoNT into a whole humanized IgG.

2. Materials and methods

2.1. Materials

YB2/0 myeloma cell line was purchased from the American Type Culture Collection (Manassas, VA), grown in RPMI-1640 with 10% fetal bovine serum (FBS) and maintained in standard T-75 flasks. For upscale production of antibody, cells were transferred to INTEGRA CELLine 350 bioreactor flasks (INTEGRA Biosciences, Ijamsville, MD). BotFab5 [2], was obtained from Dr. Peter Emanuel (Research and Technology Directorate, US Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD). Specialized human immunoglobulin (IgG) G1 mammalian expression vectors, pLC-huCk and pHC-HuCg1 [9], were obtained from Dr. Gary McLean (University British Colombia, Canada). Botulinum pentavalent toxoid (BPT) was produced by Michigan Department of Public Health (Lansing, MI). Undiluted, the BPT used in this work contained 9.4 μg/ml of BoNT A and 11.8 μg/ml of BoNT B. Normal serum IgG, conjugated and unconjugated detection antibodies, were purchased from either Sigma (St. Louis, MO) or KPL (Gaithersburg, MD).

2.2. Cloning of BotFab5 V_H and V_L

Variable heavy (V_H) and light (V_L) genes of BotFab5 were amplified by PCR using the following primers: V_H, 5'-CTA TCT ATA GCT AGC TAT CGA ATT CGT CCT TAC AAT GAA ATA CCT ATT GCC TAC G-3' and 5'-ATC TAT CTA TAA GCT TGC TGC AGA GAC AGT GAC CAG AGT-3'; V_L, 5'-TCT ATC TAT AGC TAG CAC AGC ATA AAC ATG AAA TAC CTA TTG CCT ACG-3' and 5'-TCT ATC TAT AGC GGC CGC AGT CCG TTT GAT TTC CAG CTT GGT-3'. PCR was performed using Ready-To-Go PCR Beads (Amersham Biosciences, Piscataway, NJ) un-

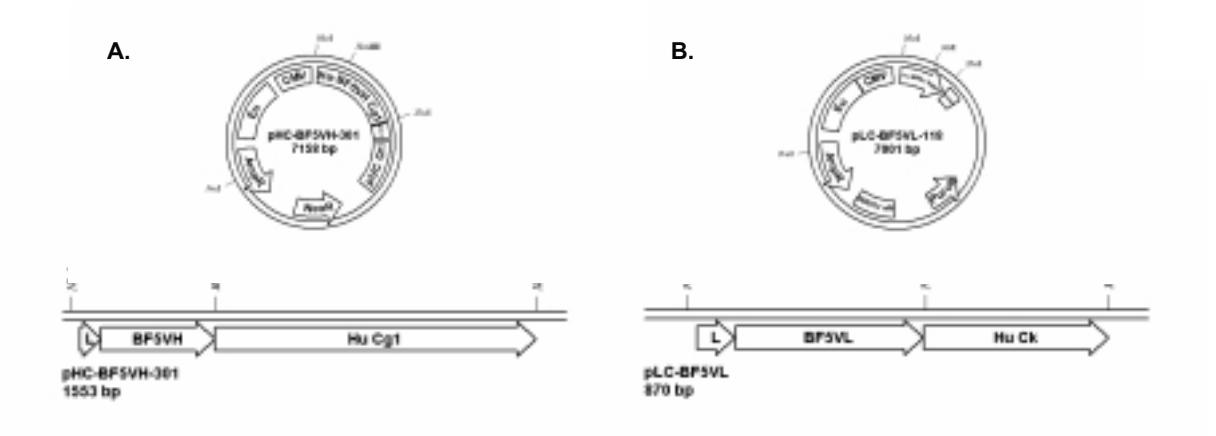

Fig. 1. Expression vectors and cloning sites for the heavy- and light-chain plasmids. (A) Schematic diagram of the heavy-chain expression vector, pHC-BF5VH-301. Eu, murine enhancer; CMV, cytomegalovirus promoter; Hu-BF5VH-Cg1, chimeric human BotFab5 gamma 1 heavy-chain gene; L, leader sequence; BF5VH, BotFab5 variable heavy gene; Hu Cg1, human gamma 1 constant gene, BDH PA, bovine growth hormone poly A sequence; pUC Ori, pUC19 origin of replication; *Neo*^{R}, neomycin resistance gene; *Amp*^{R}, ampicillin resistance gene. (B) Schematic diagram of the light-chain expression vector, pLC-BF5VL-118. Eu, murine enhancer; CMV, cytomegalovirus promoter; Hu-BF5VL-Ck, chimeric human BotFab5 LCκ gene; L, leader sequence; BF5VL, BotFab5 variable light gene; Hu Ck, human kappa constant gene; BDH PA, bovine growth hormone poly A sequence; *Puro*^{R}, puromycin-resistance gene; pBR322 Ori, pBR322 origin of replication; *Amp*^{R}, ampicillin-resistance gene.

der the following thermocycler conditions: 1 cycle of 94°C for 2 min, 60°C for 2 min, for 72°C 2 min, followed by 34 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 2 min. The V_L gene was prepared for cloning into pLC-HuCk by double *Nhe I* and *Not I* restriction digestion. The V_H gene was prepared for cloning into pHC-HuCg1 by double *Nhe I* and *Hind III* restriction digestion. V_H and V_L genes were ligated into specialized human IgG1 expression vectors, pHC-Cg1 and pLC-huCk, respectively, using Quick Ligation Kit (New England Biolabs, Ipswich, MA) to generate heavy-chain vector, pHCBF5VH-301, and light-chain vector, pLCBF5VL-118 (Fig. 1). Vector pHCBF5VH-301 contained the BotFab5 V_H gene cloned directly in front of the human *Cg1* gene and possessed a genticin resistance (*Neo*^{R}) marker. Vector pLCBF5VL-118 contained the BotFab5 V_L gene cloned directly in front of the human *C*κ gene and possessed a puromycin resistance (*Puro*^{R}) marker. Both vectors used the CMV promoter to control expression of light- and heavy-chain genes.

2.3. Transfection and selection of myelomas

Myeloma cells were chosen as the expression system based on previous literature review [11]. The cells (2.0×10^7) were sequentially transfected with the light-chain vector followed by the heavy-chain vector. The vectors were linearized by *Pvu I* digestion and 20 μg of linearized vector was transfected into the myeloma cells by electroporation (950 μF, 0.300 kV, 1 pulse, BioRad Pulser II). Cells were first transfected with the light-chain vector and grown for 2 weeks before selection with 5.0 μg/ml puromycin. Once a puromycin-resistant population emerged, culture supernatant was clarified by centrifugation and tested by ELISA for the presence of human kappa light chain (LCκ). Puromycin-resistant populations producing LCκ were grown to high density and electroporated with 20 μg of heavy chain vector as described above. Transfected cells were grown for 3 days before selection with 500 μg/ml genticin. The resulting population of cells was maintained with 5.0 μg/ml of puromycin and 500 μg/ml of genticin to yield a dually resistant population. Cells were tested by direct ELISA for the presence of whole humanized IgG antibody specific for BPT. Twelve clones were isolated by limiting dilution and expanded for further evaluation. Two clones were selected for purification by protein G HPLC.

2.4. ELISA

All assays were optimized for concentrating capture and detector antibodies individually and samples were examined in duplicate with at least three independent assays. Human LCκ was detected in a capture ELISA using goat anti-human LCκ as the capture reagent and goat anti-human LCκ HRP conjugate as the detector reagent. ELISA plates were coated with 0.5 μg/ml of anti-human LCκ (positive capture anti-

body) or 0.5 μg/ml of normal goat IgG (negative capture antibody) in phosphate buffered saline (PBS) and incubated overnight at 4°C. Plates were washed three times in an automated plate washer with wash buffer (PBS, 0.1% Tween-20 and 0.01% thimerosal) before adding sample and between all additional steps. After each step plates were incubated at 37°C for 1 hr (30 min for color development). Cell supernatants were diluted 1:2 with blocking buffer (wash buffer plus 5% dried milk) and titrated across the plate. Next detector reagent, goat-anti human LCκ HRP conjugate, was added at 0.5 μg/ml. Antibody binding was detected by adding ABTS® peroxidase substrate solution (KPL, Gaithersburg, MD) and OD_{405} was measured. OD_{405} values were adjusted by subtracting the OD_{405} value of the negative capture antibody from the OD_{405} value of the positive capture antibody.

Each whole humanized antibody, rHu-BF5-A6 and rHu-BF5-B1, were compared to the parental BotFab5 by direct and capture ELISA as described above. The antibodies were examined by direct ELISA to detect the presence of whole humanized IgG antibody specific for BPT. Plates were coated with a 1:100 dilution of BPT and cell supernatant, purified humanized antibody or normal human IgG (negative control) was added at indicated concentrations and detected with 0.5 μg/ml of goat anti-human IgG Fc-specific HRP conjugate. As a positive control, horse anti-BoNT A serum was diluted 1:100 and detected with a goat-anti horse H&L HRP conjugate. To show that each humanized antibody retained specificity to both BoNT A and BoNT B, the antibodies were tested in separate direct ELISAs for reactivity to each toxoid individually.

The humanized antibodies were also examined by capture ELISA to compare their performance to the parent BotFab5. Plates were coated with purified humanized antibody, the parent BotFab5, normal human IgG (negative control for humanized antibodies), or normal mouse IgG (negative control for Fab) at the indicated concentrations. Plates were incubated with a 1:100 dilution of the botulinum toxoid titrated 1:2 across the plate. Plates were then incubated with horse anti-BoNT A sera diluted 1:100 and detected with goat anti-horse IgG H&L HRP conjugate.

2.5. Antibody purification

Myeloma lines expressing antibodies rHu-BF5-A6 and rHu-BF5-B1 were grown in Integra celline 350 flasks (Integra Biosciences, Ijamsville, MD) using Gibco Hybridoma serum-free medium (Invitrogen) according to manufacturer's directions. Antibodies in the culture supernatant were collected, clarified, and purified on Pharmacia Hi-Trap protein G columns. Columns were washed with PBS and antibodies were eluted with glycine buffer at pH 2.5. The resulting purified antibodies were buffer exchanged into PBS. Purified product concentration was determined by microBCA (Pierce, Rockford, IL). Proteins were examined by 8–16% Tris-glycine polyacrylamide gel electrophoresis and transferred to nitrocellulose for Western analysis.

2.6. Statistics

Data are represented as the mean values $\pm$ 1 SEM. Differences between two values were tested for statistical significance ($P < 0.05$) using the two-tailed unpaired Student's t-test.

3. Results

3.1. Detection of humanized anti-botulinum IgG antibodies

The variable regions of the BotFab5 were ligated into the heavy- and light-chain IgG plasmids (Fig. 1) and YB2/0 myeloma cells were transfected to create the humanized anti-BoNT antibody, rHu-BF5-A6 and rHu-BF5-B1.

The supernatants of twelve clones were tested for the presence of human antibody that bound to BPT and expressed the Fc portion of the IgG by direct ELISA. Seven of the clones produced human IgG antibody specific for BPT (data not shown). Two of the highest antibody-producing cell lines, rHu-BF5-A6 and rHu-BF5-B1, were selected for expansion and characterization of the humanized antibody.

3.2. Characterization of humanized anti-botulinum IgG antibodies

BotFab5 can perform as both a capture and detector antibody in the ELISA [2]; therefore, we examined the humanized whole antibodies, rHu-BF5-A6 and rHu-BF5-B1, to function similarly. In the direct ELISA, the purified humanized antibodies bound toxoid from 10 μg/ml to 1.25 μg/ml (Fig. 2A). The lowest antibody concentration detecting toxoid correlated to a detection limit of 9.4 ng for botulinum toxoid A and 11.8 ng for botulinum toxoid B. In the capture ELISA, both humanized antibodies bound toxoid over a range of

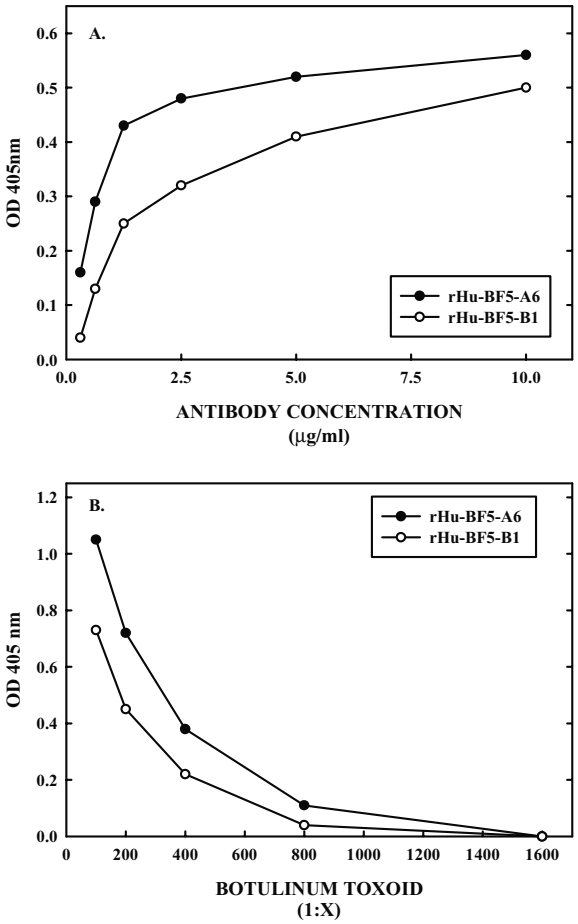

Fig. 2. Characterization of purified humanized antibodies using direct and capture ELISA. (A) For direct ELISA, plates were coated with 1:100 dilution of botulinum pentavalent toxoid (BPT) and incubated with indicated amounts of the purified rHu-BF5-A6 or rHu-BF5-B1 antibodies. Antibodies were detected with 0.5 μg/ml of goat anti-human IgG Fc HPR conjugated antibody. (B) For capture ELISA, plates were coated with 5.0 mg/ml of the rHu-BF5-A6 or rHu-BF5-B1 antibodies as the capture reagent. Plates were incubated with the indicated amount of BPT and detected with horse anti-BoNT A sera and goat anti-horse IgG HRP conjugate. Data are presented as OD values corrected for the background binding of normal human IgG. Each value represents the average of duplicate samples and data represent three independent experiments.

dilutions from 1:100 to 1:400 (Fig. 2B). The detection limit of the antibodies in the capture format was 2.3 ng for botulinum toxoid A and 3.0 ng for botulinum toxoid B.

To further confirm that the myeloma cells expressed whole IgG that was humanized and did not react to antibodies directed against the mouse species, purified antibodies were examined by immunoblot analysis. rHu-BF5-A6, BotFab5, normal human IgG and normal mouse IgG were separated by SDS-PAGE. Incubation of the nitrocellulose with anti-human LCκ HRP conjugate revealed a light-chain band in rHu-BF5-A6 but not BotFab5 compared to the human IgG positive control (Fig. 3). Likewise, incubation with anti-human IgG Fc-specific HRP conjugate demonstrated the presence of the Fc portion of the heavy chain in rHu-BF5-A6 but not BotFab5. Further, incubation with an anti-mouse HRP conjugate demonstrated no bands present in rHu-BF5-A6 antibody, but a single band in the BotFab5, supporting the full conversion from a mouse Fab to a human IgG. rHu-BF5-B1 was tested also and produced the similar results (data not shown).

3.3. Comparison of humanized IgG antibodies to mouse BotFab5

The humanized antibodies, rHu-BF5-A6 and rHu-BF5-B1, were compared to BotFab5, for their ability to bind BPT in a capture ELISA. Both rHu-BF5-A6 and rHu-BF5-B1 performed better than BotFab5 when coated on the plate at capture concentrations of 2 μg/ml (Fig. 4). The antibodies were also able to detect BPT at capture concentrations of 0.5 μg/ml while BotFab5 showed no reaction (data not shown). BotFab5 lacking a F_C portion, prevented a true comparison of the humanized antibodies to the parental BotFab5 in a direct ELISA as different conjugated antibodies as detectors would be required.

4. Discussion

Using recombinant DNA technology permits the creation of highly specific and highly selective recombinant Fabs; however, they still lack some desired characteristics of the whole human IgG molecules. BotFab5 is an anti-BoNT Fab that was selected from a phage display library prepared from mice vaccinated with the botulinum pentavalent toxoid and possesses high affinity and cross-reactivity to both BoNT A and B [2, 7]. Although useful for immunodetection as a murine Fab, BotFab5 has several drawbacks when compared with whole IgG antibodies. First, while useable in immunoassays, Fab molecules are less compatible with rapid and field formats such as ELISA and ECL that favor whole IgG antibody as reagents. Second, Fabs inherently lack the stability of whole IgG molecules. Third, Fabs are less efficient to purify and label than whole IgG. Fourth, as a murine Fab, BotFab5 cannot be used as a positive control during human serological analysis for exposure to BoNT. Therefore, the purpose

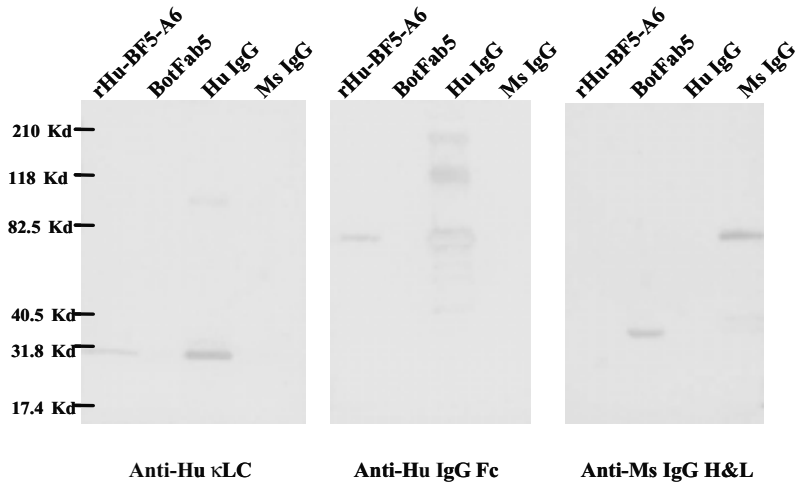

Fig. 3. Immunoblot analysis of purified humanized antibodies. Purified humanized antibody rHu-BF5-A6 (2 μg), BotFab5 (2 μg), normal human IgG (1 μg), or normal mouse IgG (1 μg) were separated by SDS-PAGE under reducing conditions. Blots were detected with anti-human LCκ, anti-human IgG Fc or anti-mouse IgG H&L. Molecular weight markers are shown on the left of each blot. Blots represent three independent experiments.

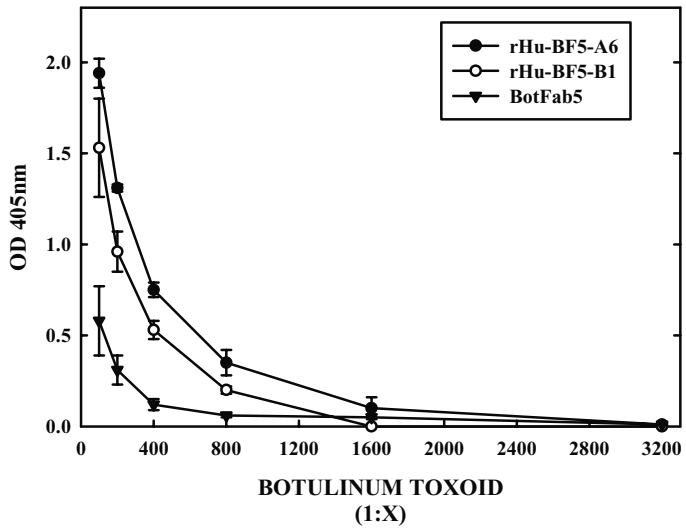

Fig. 4. Comparison of humanized IgG antibodies to mouse BotFab5. The humanized antibodies and the BotFab5 Fab were compared for detection of botulinum pentavalent toxoid (BPT) by capture ELISA. Plates were coated with 2 μg/ml of rHu-BF5-A6, rHu-BF5-B1, or BotFab5. Plates were incubated with indicated amount of botulinum toxoid and detected with horse anti-BoNT sera and goat anti-horse HRP conjugate. Data are presented as OD values corrected for the minimal background binding of normal human IgG or normal mouse IgG. Each value represents the average of duplicate samples and data represent three independent experiments.

of these experiments was to demonstrate the conversion of a recombinant Fab into a whole humanized IgG that would perform equal to or better in diagnostic assays and could be used as a human positive control.

These results demonstrate the ability to convert a mouse Fab into a chimeric murine-human IgG antibody. Cloning the variable regions of the Fab into a two-vector system expressing heavy and light chains, it was possible to produce humanized antibodies in cell culture. The new murine-human chimeric IgG antibodies retained their binding specificity to botulinum toxoid and actually demonstrated improved function as capture antibodies. Structural comparison of the molecules suggested the improved function of the whole antibody may actually be greater than demonstrated in our assays. Whole antibodies weigh approx-

imately 150 kD and Fabs about 50 kD. Therefore on a molar basis, there are 3 antigen binding sites in the Fab preparations versus 2 binding sites per whole IgG molecule. Since the concentrations of each preparation used to coat the ELISA plates were equivalent, the recombinant whole antibody would have 50% fewer antigen binding sites than the Fab, yet produced equivalent results. Therefore, fewer whole antibody molecules were able to bind the same or more target than the Fab, an apparent improved binding affinity (or avidity).

The improved characteristics of the whole antibody can also be due to increased stability of the molecule from the pairing of the constant domains. Previous work showed an increase in affinity of whole IgG molecules compared to scFv due to increased stability [12]. The larger molecule may also allow for more directed orientation in the ELISA platform creating a more favorable interaction with the antigen. In addition, some improvements in binding may be attributed to the bivalent nature of a whole antibody versus a Fab [13]. Studies have suggested that linking monovalent antibody fragments to create bi or multivalent fragments improves binding avidity similar to whole antibodies [13,14]. However, it is unlikely that the improvement in detection we observed was due to a change in the antibody specificity since humanized whole antibodies, rHu-BF5-A6 and rHu-BF5-B1 contained the same variable region or antigen-binding domains.

The ability to convert a Fab to a whole human IgG can have numerous applications. One use, as previously mentioned, is the ability to create human antibodies to serve as controls in the testing of human sera. Many diagnostic laboratories do not have collections of human sera from patients infected with rare infectious diseases that can be used as positive controls. This technology will permit the screening of mouse Fab libraries against various infectious disease targets, with further conversion into human IgG [15]. Conceivably, human-positive controls for all infectious diseases could be made available by using recombinant DNA technology.

In addition to positive controls, whole human IgGs could be made from mouse Fabs that display neutralizing ability. Mouse neutralizing antibodies typically lack therapeutic effect in humans due to the immune reaction they elicit [16,17]. However, recent work in the area of chimeric antibodies has shown that humanizing animal antibodies can lessen the host immune response and be beneficial for controlling disease progression [18–20]. While the humanized IgG created in this work was probably not neutralizing, being directed against hemagglutinin, it may be possible to convert a mouse neutralizing antibody into a human neutralizing antibody for therapeutic testing.

The technology discussed in this paper relies on the proper and accurate cloning of the variable regions from the mouse Fab. Various labs have published PCR primers for cloning of antibody variable regions [8,21, 22]. Using these primers it should be possible to clone these regions from any whole antibody, regardless of the species or class of antibody. Therefore, the technology presented in this paper should be instrumental for developing protocols to class switch or species switch any antibody. It is foreseeable to create one antibody, with outstanding detection ability, and use it to generate a whole group of reagents. One mouse IgM could be converted into a mouse IgG, a human IgG and a human IgM [9] allowing for the production of human controls without any exposure of humans to dangerous pathogens.

In conclusion, the ability to convert antibody fragments into whole human antibodies can dramatically improve their utility for diagnostic applications. Not only does the whole IgG format overcome several shortcomings associated with antibody fragments, it also allows for the creation of human serological controls where none may exist. This technology, coupled with the increasing sophistication of antibody display and engineering technologies could generate a wide variety of high quality antibody reagents for the detection and serological analysis of biological agents.

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